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Autotrophic Carbon Fixation by the Chemoautotrophic Symbionts of *Riftia pachyptila*

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Abstract. Preparations of trophosome tissue from *Riftia pachyptila* containing viable endosymbiotic bacteria were incubated with several substrates under a variety of conditions to characterize the symbionts physiologically. Of all the potential substrates tested, only sulfide stimulated carbon fixation by the trophosome preparations; neither hydrogen, ammonia, nor thiosulfate were effective. Trophosome preparations did not oxidize ^{14}C-methane to either ^{14}C-organic compounds or $^{14}CO_2$, nor did they reduce acetylene under the conditions tested. Carbon fixation by the endosymbionts appears barotolerant. The symbionts require both sulfide and oxygen to fix carbon through autotrophic pathways, but are inhibited by free oxygen and by sulfide concentrations in the 300 μM range. Maximal rates of carbon fixation were documented in incubations in dilute *Riftia* blood, which protects the symbionts from the inhibitory effects of free sulfide and oxygen while providing them with an abundant pool of both substrates, bound by the vestimentiferan hemoglobins.

Introduction

Symbioses between marine invertebrates and endosymbiotic chemoautotrophic sulfur bacteria were first discovered at deep sea hydrothermal vents (Cavanaugh *et al.*, 1981; Felbeck, 1981). Similar associations are now well documented in several phyla of worms and in five molluscan orders found in a variety of habitats where redueed chemical species are present (Fisher, 1990). In all groups except the Vestimentifera, symbionts are housed in close proximity to the external environment. They are found in gill cells (most molluscs), in internal cells in very small animals (pogonophorans), or extracellularly, on the surface of the animal (oligochaetes, alvinellids, and thyasirids). These symbionts are therefore in close contact with the necessary metabolites carbon dioxide, sulfide, and oxygen (Fisher, 1990). In contrast, the vestimentiferans examined to date harbor abundant endosymbiotic, sulfide-oxidizing, chemoautotrophic bacteria in an internal organ—the trophosome. This organ is highly vascularized and is located in the trunk of the worm; it has no close connections to ambient seawater (Jones, 1981).

The trophosome comprises about 15% of the wet weight of the hydrothermal vent tubeworm, *Riftia pachyptila*, and hemoglobin-containing vascular and coelomic fluids account for at least another 30% (Childress *et al.*, 1984). The bacterial volume is between 15 and 35% of the total volume of the trophosome, and estimates of bacterial density range from 3.7 to 10×10^9 cells/g trophosome (Cavanaugh *et al.*, 1981; Powell and Somero, 1986). Because this organ is located in a coelomic cavity in the trunk of the animal, metabolites must be transported to the bacteria through the circulatory system. Vestimentiferan hemoglobins, found in both the hemolymph and coelomic fluid, bind hydrogen sulfide and oxygen independently and reversibly, preventing spontaneous oxidation of the sulfide while transporting it to the trophosome for use as an electron donor by the chemoautotrophic endosymbionts (Arp and Childress, 1983; Childress *et al.*, 1984; Fisher and Childress, 1984; Arp *et al.*, 1987). The high affinity of the hemoglobins for sulfide also protects the animal cytochrome *c* oxidase system from poisoning by this potentially toxic molecule (Powell and Somero, 1983, 1986).

Few studies have been done on the physiology of vestimentiferan symbionts, most likely because it is difficult

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to obtain living material. Belkin *et al.* (1986) demonstrated that the symbionts from several individuals of *R. pachyptila* used sulfide, and not thiosulfate, as an electron donor to fuel chemoautotrophic carbon fixation. Fisher *et al.* (1988a) reported sulfide stimulated carbon fixation by the symbionts of another vestimentiferan. Wilmot and Vetter (1990) recently reported that only exogenously supplied sulfide (not thiosulfate or sulfite) is oxidized by the symbionts, and that oxygen consumption by both trophosome preparations and partially purified symbionts is not inhibited by atmospheric levels of oxygen, or by sulfide concentrations below 2 m*M*. All of the vestimentiferans tested (including numerous individuals of *R. pachyptila*) have contained appreciable activities of RuBP carboxylase/oxygenase, ATP sulfurylase, and adenosine-5′-phosphosulfate (APS) reductase in their trophosomes indicating that the symbionts are chemoautotrophic sulfur oxidizers (Felbeck, 1981; Felbeck *et al.*, 1981; Brooks *et al.*, 1987; Fisher *et al.*, 1988b; Cary *et al.*, 1989). While one study suggested that *R. pachyptila* trophosome homogenates could oxidize methane (Fisher and Childress, 1984), another demonstrated that the intact symbiosis did not take up methane and that the hemolymph lacked a binding protein for methane (Childress *et al.*, 1984). In addition, stable carbon isotope studies have led some investigators to suggest that methane may be oxidized by some vestimentiferans (Kulm *et al.*, 1985).

The role of vestimentiferan blood in protecting the endosymbionts of an unnamed hydrocarbon-seep escarpid (phylum Vestimentifera) from the toxic effects of hydrogen sulfide, while supplying the endosymbionts with a large pool of bound hydrogen sulfide, has recently been reported (Fisher *et al.*, 1988a). Although carbon fixation by the symbionts of *R. pachyptila* is dependent on the availability of oxygen (Belkin *et al.*, 1986), investigators have only speculated about the role of vestimentiferan blood in protecting the symbionts from oxygen inhibition of carbon fixation, while simultaneously providing a large pool of this required substrate, (Childress, 1987; Fisher *et al.*, 1988a).

Here we report the results of several experiments carried out with trophosome preparations from *Riftia pachyptila*. A variety of potential electron donors were tested for suitability as an electron donor for the endosymbionts. Methane oxidation by the endosymbionts was investigated using both ^{14}C methane and $NaH^{14}CO_2$. The symbionts' ability to fix molecular N_2 was tested by the acetylene reduction method under a variety of conditions. Barotolerance of symbiont autotrophy was studied in incubations of trophosome preparations at high pressure. Finally, sensitivity of symbiont carbon fixation to free oxygen and sulfide, and the role of vestimentiferan blood in providing these substrates at appropriate activities for maximal rates of autotrophic carbon fixation, was investigated.

Materials and Methods

Experimental material

The *Riftia pachyptila* used in these experiments were collected during three cruises: two to the Galapagos Rift (Galapagos 1985, and Galapagos 1988), and one to 13°N on the East Pacific Rise (Hydronaut in 1987). On all cruises, the animals were collected by submersible (either ALVIN or NAUTILE) and brought to the surface in a temperature insulated container. Upon recovery, the animals were placed in fresh, chilled seawater and transferred to a refrigerated van for processing. Most of the animals used in this study were dissected immediately after recovery; some were transferred to flow-through pressurized aquaria (Quetin and Childress, 1980) for short term maintenance (less than three days) before being used.

The experiments reported here were conducted on tissue from living worms. Trophosome tissue is extremely fragile and deteriorates rapidly when the organ is even slightly damaged. When damaged, the tissue begins to take on a fuzzy pink appearance. In the early stages, this is just visible between the lobes of the trophosome tissue. This "fuzzy pink" effect may be due to the effects of lysozymes on the blood, which is normally a deep red color. Tissue from damaged individuals has always shown very low rates of carbon fixation in our studies. About 25% of externally undamaged animals (especially larger individuals) dissected immediately upon recovery contained a substantial portion of visibly degraded trophosome tissue. The incidence of degradation, and its intensity, increased dramatically in animals held at ambient pressure for even a few hours before dissection. No damaged tissue was used in this study.

Preparation of Riftia *saline*

Riftia saline was prepared based on the average concentrations of inorganic salts measured in both vascular and coelomic fluids. The saline was titrated to pH 7.5 with NaOH before use. One liter of *Riftia* saline contains: 20.48 g NaCl (0.4 *M*); 0.194 g KCl (2.6 m*M*); 6.22 g $MgCl_2*6H_2O$ (30.6 m*M*); 1.65 g $CaCl_2*2H_2O$ (11.2 m*M*); 4.53 g Na_2SO_4 (31.9 m*M*); and 11.915 g HEPES (50 mM) (Fisher *et al.*, 1988a).

Preparation and analysis of Riftia pachyptila *blood*

The *Riftia pachyptila* blood used in these experiments was collected from living worms and, unless otherwise specified, was kept frozen at either −20 or −70°C until used. This blood was a mixture of coelomic fluid and

hemolymph from a number of individuals. Bound sulfide was removed from the blood by acidifying to pH 5.5 with HCl, and purging the chilled blood (7°C) with a stream of nitrogen for 24 h. The blood was diluted with vestimentiferan saline solution before use in the trophosome homogenate incubations.

In two sets of experiments, fresh blood was used in the incubations. Blood from freshly collected *R. pachyptila* was neither acidified to remove bound sulfide, nor frozen, before use in the experiments in which free oxygen was varied in blood incubations (Fig. 2C and Table II). For these experiments, the chilled blood (pH 7.5) was saturated with oxygen by stirring under a stream of air for 15 min and then stripped of free (unbound) oxygen by stirring under a stream of nitrogen for an additional 45 min before use. Because it is difficult to strip the hemoglobins of oxygen, this treatment has little effect on the amount of oxygen bound by the hemoglobin. The result, therefore, is blood with the hemoglobin virtually saturated with oxygen, but containing very low concentrations of free oxygen in solution. This blood stock was then loaded into a gas-tight syringe, and added to incubation syringes containing saline solution with various concentrations of oxygen to generate variable concentrations of free oxygen in the experimental syringes. In the experiments shown in Figure 5 (testing symbiont sulfide sensitivity), fresh coelomic fluid from worms maintained in pressure aquaria in the absence of sulfide was used because it contained very low levels of bound sulfide (28 μM), and therefore did not require the acidification treatment to remove bound sulfide.

The heme content of the blood mixture was determined from the absorbance of a cyanomet hemoglobin derivative (Tentori and Salvati, 1981). We determined the capacity of separate aliquots of the bloods for sulfide by equilibrium dialysis of the blood in 30 mM citric acid phosphate buffer at 7°C and pH 7.5 (Arp and Childress, 1983). Samples of the blood in dialysis tubing were allowed to come to equilibrium (24 h) with sulfide in the dialysate (1 mM). The concentrations of sulfide in both blood and dialysate were analyzed with a gas chromatograph (Childress *et al.*, 1984). The difference between the sample sulfide and the dialysate sulfide was the amount of sulfide bound.

Preparation and analysis of Calyptogena magnifica *serum*

Dilute *Calyptogena magnifica* serum was used as an incubation medium in several experiments because the clam serum binds sulfide but not oxygen (Arp *et al.*, 1984). The clam serum was collected during the "Galapagos 1985" expedition and was used in experiments conducted during the "Hydronaut" expedition. Clam blood was collected from freshly recovered clams and centrifuged for 3 min in a bench top centrifuge at about 2000 × *g*. Dissection of the clams, collection of the blood, and centrifugation was all conducted in a refrigerated van (6–8°C). The serum was transferred to plastic scintillation vials, frozen at −20°C on board ship, then transferred to −70°C for storage in the laboratory. Before use, the serum was concentrated and cleared of precipitates by dialysis against distilled water for 16 h in a vacuum concentrator. Binding capacity of the serum for sulfide was determined as above for the *Riftia* blood. The binding capacity of the concentrated serum was 8.43 mM, and it was diluted with *Riftia* saline to a binding capacity of 2.0 mM before use as a serum stock in the trophosome incubations.

Preparation of the trophosome homogenates

Trophosome tissue containing symbiotic bacteria was dissected from living *Riftia pachyptila* and separated from the major blood vessels and gonads also present in this organ. A portion of the tissue (0.4–1.0 g) was blotted for a few seconds on a paper towel to remove excess blood, weighed on a motion compensated shipboard balance system (Childress and Mickel, 1980), and then submerged in about 7 ml of chilled, deoxygenated (nitrogen purged) *Riftia* saline solution (Fisher *et al.*, 1988a). The tissue was gently homogenized for 5–10 s in a chilled, loose fitting Dounce type ground glass tissue homogenizer (2–4 passes), to rupture most of the bacteriocytes and disperse the symbionts. The homogenate was diluted to either 30 or 60 ml with additional deoxygenated saline and loaded into one or two glass 30-ml syringes, equipped with three-way valves, containing marbles to mix the homogenate. This entire procedure takes between 5 and 10 min and, except for weighing, was conducted in a refrigerated van (6–8°C). A portion of this homogenate (0.1 ml) was fixed in 0.9 ml of 3% glutaraldehyde in 0.1 M phosphate-buffered 0.35 M sucrose (pH 7.35) for later examination using epifluorescence microscopy (Hobbie *et al.*, 1977).

For the experiments in which the effect of free oxygen concentration on carbon fixation was examined, the weighed tissue was transferred to a glove bag (in the refrigerated van), and the homogenate was prepared and loaded into the 30-ml syringe under a nitrogen atmosphere.

$NaH^{14}CO_3$ incubations

The incubations were conducted in 10-ml glass syringes (except the Galapagos 1985 experiments, which were conducted in disposable 10-ml plastic syringes) fitted with low dead volume teflon valves. Experiments were conducted in a refrigerated van that was main-

tained between 6 and 8°C (the incubation temperature during an experiment was constant, but the temperature inside the van varied slightly from day to day). Prior to preparation of the homogenate, between 6 and 18 syringes were loaded with the incubation media and substrate concentrations appropriate for a given experiment. All media and substrate stock solutions were titrated to pH 7.5 before use. The incubation media contained variable amounts of *Riftia* blood or *Calyptogena magnifica* serum diluted with *Riftia* saline, or else *Riftia* saline alone. The various sulfide concentrations used in the experiments were generated in the experimental syringes by adding appropriate amounts of a sulfide stock solution (7 to 15 m*M*) to the experimental syringes, using a three-way valve on the stock syringe. Similarly, variable oxygen concentrations were generated in the experimental syringes by introducing a mixture of saline stock solutions of variable oxygen concentrations (also contained in syringes and introduced through three-way valves). Stock solutions of methane and hydrogen were prepared by bubbling a saline solution with the appropriate gas. The stock solutions of thiosulfate and ammonia were prepared from sodium thiosulfate and ammonium chloride, respectively. Sulfide, methane, and inorganic carbon concentrations in the blood and saline stock solutions, and oxygen concentration in the saline stocks, were determined using a gas chromatograph (Childress *et al.*, 1984). (O_2 concentrations were not directly quantifiable in blood by this method.) Ammonium concentration in the stock solution was verified by flow injection analysis (Willason and Johnson, 1986). To confirm that stimulated carbon fixation was through autotrophic pathways, 10 m*M* DL-glyceraldehyde (a feedback inhibitor of RuBP carboxylase-oxygenase; Stokes and Walker, 1972) was added to one syringe in most of the experiments (Fisher *et al.*, 1988a).

To start the incubations, $NaH^{14}CO_3$ was added to the trophosome preparation, and 2.5 ml of the labeled preparation was drawn into each of the syringes, which already contained incubation media with the desired levels of sulfide, oxygen, or other substrates. The final concentrations of $NaH^{14}CO_3$ used in these experiments ranged from 0.1 to 1.0 μCi/ml depending on the experiment. Activity of $NaH^{14}CO_3$ in the syringes was determined by scintillation counting of replicate samples of the trophosome preparation stabilized with hyamine hydroxide. After all the experimental syringes had been filled and the contents mixed (a process that took between 4 and 7 min), replicate samples (0.1 ml) were removed from each syringe and acidified for scintillation counting of the fixed carbon. Replicate samples (0.1 ml) were similarly removed and assayed from each syringe at 10- to 20-min intervals for the next 60 to 80 min. Carbon fixation rates were calculated, following the methods of Strickland and Parsons (1972), from the measured concentration of inorganic carbon in the incubation media, the measured specific activity of that inorganic carbon pool, and the amount of acid-stable ^{14}C found in the samples at each time point.

Fixation rates were calculated after subtracting first sample values from the values measured at the later time points. This method of analysis compensated for the variable rates of carbon fixation before, and during, the first few minutes that the preparations were exposed to the substrates in the experimental syringes (Fisher *et al.*, 1988a). Trophosome preparations that failed to show a carbon-fixation rate greater than 0.2 μmol/g/h under any experimental conditions were considered to contain, at best, only marginally viable symbionts, and no data from these preparations are reported here.

Incubations under pressure

In the experiments designed to test the effect of pressure on the carbon fixation rate by the trophosome preparations, identical paired syringes were prepared, and one of each pair was incubated in a pressure vessel at 100 atm. One hundred atm was considered a sufficient pressure because this pressure supports extended survival of the tubeworms that cannot survive at 1 atm (Childress *et al.*, 1984). For pressure incubations, the glass syringes were suspended in an acrylic pressure vessel (Quetin and Childress, 1980), with a fine teflon tube extending from a luer lock fitting on the syringe to the outside through high pressure valves. Thus, samples could be taken from the syringes without releasing the pressure around the incubations. The pressure vessel was inverted repeatedly so that the marbles in the syringes would mix the samples.

Rate calculations

Determination of the appropriate carbon fixation rate for analysis was often problematic because, under some conditions, the fixation rates were distinctly non-linear over the course of the incubation. These situations were handled as follows: if the carbon fixation showed no systematic increases or decreases during the incubation (did not appear to increase or decrease significantly as a function of time), then rates were calculated by linear regression of all data points. This was the situation in most of the incubations in *Riftia* blood, and some of the saline experiments. If the rates in all of the syringes during an experiment decreased over time, then rates were calculated from the same portion of the experiment for all syringes (the first two, three, or four time points). In some of the saline and serum incubations, where either sulfide or oxygen was low, the rates decreased as the limiting substrate was exhausted, and the rates were therefore cal-

culated from the first two or three points. In a few incubations where the substrate (oxygen or sulfide) was initially present in slightly inhibitory concentrations, the rates increased over the course of the incubations as the substrate was depleted. The rates in these experiments were also calculated from the first few time points. Examples of these situations can be seen in Fisher *et al.* (1988a). The specific experiments in which rates were calculated from less than all five points are indicated in the results section. Data were always treated consistently within an experiment.

^{14}C-methane incubations

Trophosome samples from two individuals of *R. pachyptila* were also tested for the ability to use methane as a carbon or energy source. $^{14}CH_4$, synthesized microbiologically as described by Daniels and Zeikus (1983), having a specific activity of 4×10^5 dpm/μl, was used as a tracer for methane oxidation by trophosome preparations. Contamination of the labeled methane was less than 0.01% as determined by gas proportion counting.

Trophosome homogenates were prepared as described above, and 10 ml of the homogenate was placed in each of six 35-ml serum vials. The headspace was flushed with a stream of nitrogen for 1 min, then the vials were capped with butyl rubber stoppers and crimped with aluminum seals. The headspaces over the vials were adjusted using a gas-tight syringe to remove nitrogen and inject air and methane to produce the desired concentrations of dissolved oxygen ($\sim$100 μM) and methane, as estimated from Bunsen coefficients. Two methane concentrations were tested in each experiment: 10 and 100 μM in the first experiment and 5 and 10 μM in the second. $^{14}CH_4$ tracer stock (500 μl) was added to each vial 30 min after injecting the cold methane to initiate the incubations. Duplicate samples and a formalin-killed control were incubated for each methane concentration. The incubations were terminated 1 h after introducing the labeled methane by adding 200 μl of 5 *N* NaOH to each. The seals were then removed from each vial and the stoppers replaced by another, with a piece of Whatman #1 filter paper soaked with 100 μl of phenethylamine suspended from a wire into the headspace. The vials were re-sealed, and 500 μl of concentrated H_2SO_4 was added to each vial by injection. The filters were allowed to absorb the CO_2 released from the liquid for 24 h, and then removed and placed in 10 ml of 3a70 fluor (National Diagnostics) and counted in a liquid scintillation counter. Replicate 100-μl samples of the homogenate were degassed and assayed for acid stable ^{14}C by liquid scintillation counting.

Results

Potential symbiont substrates

The results of experiments conducted during the Galapagos 1985 expedition should be regarded as preliminary because these techniques were developed during this expedition. Nine sets of experiments were conducted during this expedition, with preparations of trophosome material from nine individuals of *Riftia pachyptila*. In each of these experiments, six to ten syringes were run simultaneously. Only hydrogen sulfide (among electron donors tested in these experiments) significantly stimulated carbon fixation: by as much as 140% in saline, and by as much as 880% when incubations were conducted in 50% *Riftia* blood. (Control syringes containing blood but no trophosome preparation did not fix carbon.) Considerably higher rates of carbon fixation (ten- to twenty-fold) were found when the incubations were conducted in dilute *Riftia* blood as compared to incubations in saline alone. Neither methane (140–500 μM, 10 incubations, 5 worms), hydrogen ($\sim$⅓ saturated, 5 incubations, 3 worms), ammonia (50 and 100 μM, 6 incubations, 3 worms), nor thiosulfate (0.05 and 0.5 mM, 6 incubations, 3 worms) had a significant effect on the rate of carbon fixation in these experiments. All of these comparisons were conducted under both aerobic (0.1–0.2 mM oxygen) and micro-aerobic (0.02–0.04 mM oxygen) conditions.

Trophosome tissue from two individuals of *R. pachyptila* tested during the Galapagos 1988 expedition did not oxidize ^{14}C-methane to either ^{14}C-organic compounds or $^{14}CO_2$.

Trophosome tissue from two individual worms was tested for the ability to fix N_2 by the acetylene reduction method during the Galapagos 1985 expedition. Tissue from both individuals was tested under both aerobic and micro-aerobic conditions in both saline and dilute *Riftia* blood, with and without sulfide (100 μM) as an energy source. Results were negative (no appearance of ethylene) in all 12 incubations, which lasted either 9 or 16 hours.

Effects of pressure on carbon fixation

Seven experiments, conducted during the Hydronaut expedition, were designed to test the effects of pressure on carbon fixation by *Riftia pachyptila* trophosome preparations (Table 1). The carbon fixation rates for the incubations conducted in saline (3 experiments) were calculated from the first three points only, because the rates decreased significantly during the incubations (Fig. 1a). Similar decreasing rates were observed during many of the other incubations in saline, but the phenomenon was most pronounced in these incubations; a possible cause was the increased time involved in beginning the experiments in pressure vessels, an additional 15–20 min after preparing the homogenate. No significant rate decrease was observed in the blood incubations during the experiments (Fig. 1B). Typical results of this study are

Table I

Effects of pressure on carbon fixation rate of Riftia pachyptila *trophosome tissue*

Exp.	Pres. (ATM)	[Sulfide] (μM)			Carbon fixation rates [μmol $^{-1}$ h $^{-1}$ (r^2)]				Ratio of P/A*		
		Low	Med.	High	Low	Med.	High	Inhib.**	Low	Med.	High
S 1	100	35	65	260	2.40 (1.0)	2.49 (.72)	2.80 (.83)		1.26	1.00	1.35
	1	35	65	260	1.91 (.63)	2.49 (.62)	2.07 (.18)				
S 2	100	25	50	200	1.63 (.25)	2.19 (.27)	1.33 (.16)		1.55	1.60	1.06
	1	25	50	200	1.05 (.28)	1.37 (.53)	1.26 (.31)	0.49 (.22)			
S 3	100	30	60	230	3.82 (.25)	3.38 (.15)	2.32 (.08)		1.12	1.02	1.63
	1	30	60	230	3.41 (1.05)	3.32 (.60)	1.42 (.68)	1.27 (.20)			
B 1	100	55	230	570	5.8 (.32)	8.7 (1.33)	7.7 (.77)		0.69	0.94	0.91
	1	55	230	570	8.4 (.26)	9.3 (1.12)	8.5 (.71)	2.7 (.17)			
B 2	100	85	335	840	9.3 (.56)	10.3 (.67)	5.0 (.56)		0.89	1.00	0.98
	1	85	335	840	10.4 (.66)	10.3 (1.65)	5.1 (.91)	5.0 (.35)			
B 3	100	110	395	1060	7.22 (.57)	6.11 (.87)	2.46 (.32)		0.99	1.04	1.08
	1	110	395	1060	7.30 (.72)	5.90 (.74)	2.27 (.19)	3.83 (.18)			
B 4	100	110	395	1060	8.5 (.11)	9.0 (.40)	2.5 (.12)		0.90	0.98	1.19
	1	110	395	1060	9.4 (.26)	9.2 (.39)	2.1 (.12)	3.0 (.08)			

Standard errors of the slopes of the linear regressions are presented in parentheses next to the rates. Linear regressions (and rates) were calculated from the first three time points only for the saline incubations.

S—experiments conducted in *Riftia* saline.

B—experiments conducted in dilute *Riftia* blood (binding capacity about 330 μM).

Low, Med., and High are related to each other within an experiment and refer to the sulfide concentrations given in the table for each experiment.

* P/A is the ratio of the rate at 100 ATM to the rate at 1 ATM in that experiment.

** The inhibitor (10 mM D,L-glyceraldehyde) was added to a syringe containing the "Med." level of sulfide in the saline experiments and "Low" level of sulfide in the blood experiments.

depicted in Figure 1. In the saline incubations, the carbon fixation rates under pressure were the same or slightly higher (averaging 30% higher) at all three sulfide concentrations (Table I), but there were no significant differences in the slopes of the linear regressions (rates) in pairwise comparisons within experiments. When the incubations were conducted in blood, carbon fixation rates were slightly lower under pressure. The only significant differences in the slopes of the paired pressure and ambient incubations occurred in run B1 at 55 μM sulfide (Table I).

Effects of oxygen concentration on carbon fixation rates

Ten experiments conducted during the Hydronaut expedition were designed to determine the effect of free oxygen on the carbon fixation rates of trophosome preparations. The results of three experiments conducted in saline and four experiments conducted in 8% *Calyptogena magnifica* serum were substantially the same (Table II). Maximal fixation rates in saline and dilute clam serum were recorded at initial oxygen concentrations between 72 and 154 μM, with higher oxygen concentrations significantly inhibiting carbon fixation. These rates were calculated from the first two or three points when the carbon fixation pattern was distinctly non-linear, as it was at all limiting oxygen concentrations (see Fig. 2A). The difference between limitation and inhibition was evident from the shape of the curves at the different oxygen concentrations (compare the fixation patterns at 53 and 160 μM O_2 in Fig. 2A). The rates presented in Table II for the saline and clam serum experiments are higher than the actual measured inorganic carbon incorporation rates for the 60 to 70 min incubations because they were calculated from the initial, linear portion of the curves (see Fig. 2A).

When incubations were conducted in dilute *Riftia* blood (with O_2 saturated hemoglobin), none of the incubations appeared oxygen limited (the carbon fixation rates were linear at all oxygen concentrations throughout the experiments, Fig. 2B), and oxygen inhibition of carbon fixation was evident at much lower free (unbound) oxygen concentrations than in the saline and clam serum experiments (data shown in Fig. 2B are typical of the blood incubations). This is most easily visualized in Figure 3, where all of the experiments are summarized. Each point in Figure 3 represents a rate calculated from one incubation, and the rates are presented as a percent of the maximal rate observed with that preparation. The maximum rates calculated from each experiment are given in Table II.

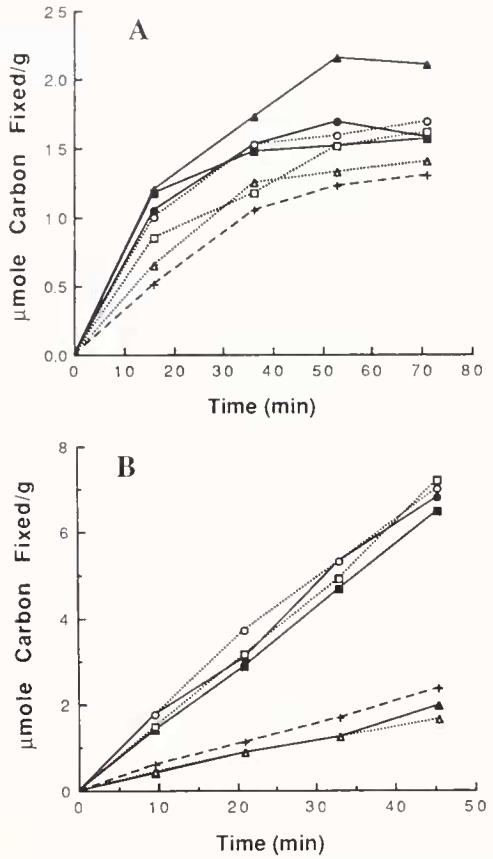

Figure 1. Effects of 100 atm pressure on carbon fixation by *Riftia pachyptila* trophosome preparations at three sulfide concentrations. Open symbols and dotted lines represent data from incubations at ambient pressure and closed symbols represent data from incubations at 100 atm: (A) incubations in *Riftia* saline without blood (Saline 1, Table 1): squares, 35 μM initial sulfide; circles, 65 μM initial sulfide; triangles, 260 μM initial sulfide; +, 1 atm, 65 μM initial sulfide and 10 mM D,L-glyceraldehyde. (B) Incubation in dilute *Riftia* blood with binding capacity of about 330 μM (Blood 4, Table 1): squares, 110 μM initial sulfide; circles, 395 μM initial sulfide; triangles, 1060 μM initial sulfide; +, 1 atm, 110 μM initial sulfide and 10 mM D,L-glyceraldehyde.

Effects of sulfide concentration on carbon fixation rates

Experiments were conducted during the 1988 Galapagos expedition to determine the optimum concentrations of sulfide for chemoautotrophic carbon fixation by *Riftia pachyptila* trophosome preparations. The results of the four experiments conducted in saline, in which the maximal carbon fixation rates were greater than 1 μmole $g^{-1}\,h^{-1}$, are shown in Figure 4. Remember that the actual concentrations during the incubation are lower than the initial concentrations presented in the figure legends due to spontaneous oxidation of sulfide in the saline incubations. The inhibitory levels of sulfide in the saline incubations are, therefore, maximum values. The initial sulfide concentrations that were maximally stimulatory to carbon fixation by the trophosome preparations incubated in saline ranged from 250 to 350 μM in the four experiments (Fig. 4). The trophosome preparations were sulfide limited at initial concentrations below 175 to 250 μM, and sulfide inhibition became apparent at concentrations of 350 to 500 μM in these experiments (Fig. 4).

Ten experiments were conducted in various concentrations of *Riftia* blood that had been collected during the Hydronaut expedition, stored at −20°C for several months, and treated as described in the methods section to remove bound sulfide. We consider the results of these experiments preliminary because subsequent analysis of the blood used indicated that it did not have the affinity,

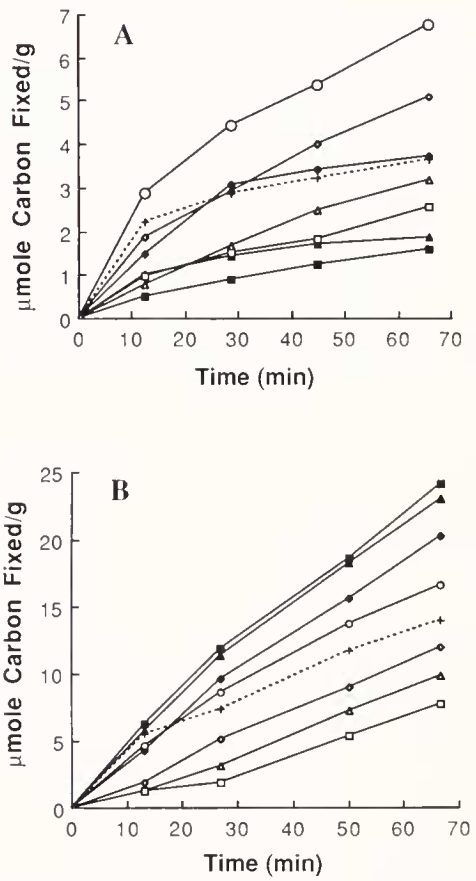

Figure 2. Effects of free oxygen concentration on carbon fixation by *Riftia pachyptila* trophosome preparations. (A) Incubations in *Riftia* saline without blood: closed square, trace oxygen; closed triangle, 27 μM initial oxygen; closed diamond, 53 μM initial oxygen; open circle, 107 μM initial oxygen; open diamond, 160 μM initial oxygen; open triangle, 213 μM initial oxygen; open square, 267 μM initial oxygen; plus with dotted line, 107 μM initial oxygen and 10 mM D,L-glyceraldehyde. (B) Incubations in 18% *Riftia* blood and balance saline: closed square, trace unbound oxygen; closed triangle, 47 μM initial unbound oxygen; closed diamond, 94 μM initial unbound oxygen; open circle, 140 μM initial unbound oxygen; open diamond, 187 μM initial unbound oxygen; open triangle, 234 μM initial unbound oxygen; open square, 292 μM initial unbound oxygen; plus with dotted line, 47 μM initial unbound oxygen and 10 mM D,L-glyceraldehyde.

Table II

Effect of oxygen concentration on carbon fixation by Riftia pachyptila *trophosome preparations*

	Incubation conditions			Maximum rate of C fixation	
Run #	Media	$[H_2S]$ (μM)	$[\Sigma CO_2]$ (mM range)	Rate (μmol g^{-1} h^{-1})	$[O_2]$ (μM)*
S 1	Saline	60	2.51–2.56	13.73	107
S 2	Saline	100	2.58–2.59	11.60	107
S 3	Saline	65	2.01–2.41	1.20	90
CS 1	8% Serum	160	1.16–1.35	5.63	154
CS 2	8% Serum	160	1.42–1.48	3.60	108
CS 3	8% Serum	110	1.71–1.83	3.31	72
CS 4	8% Serum	110	1.71–1.83	13.80	108
RB 1	18% Blood	140	3.58–3.61	28.03	Trace
RB 2	18% Blood	140	3.58–3.61	20.48	Trace
RB 3	9% Blood	85	2.27–2.30	13.47	38

S—*Riftia* Saline; CS—*Calyptogena magnifica* Serum; RB—*Riftia pachyptila* Blood.

* Oxygen concentration in the syringe which produced the rate of carbon fixation given in the previous column; oxygen concentrations in blood incubations refer to free oxygen, the hemoglobins are saturated with oxygen in all three experiments.

or capacity, for sulfide that heme content and experience indicates it should (Arp *et al.*, 1987; Fisher *et al.*, 1988a). Although the results were variable with respect to the absolute levels of sulfide that were inhibitory to carbon fixation in the different experiments, carbon fixation was never inhibited by sulfide levels below the estimated blood binding capacity.

When it became apparent that this frozen, treated blood was damaged, two experiments were conducted using fresh coelomic fluid from two worms that had been maintained in pressure aquaria without sulfide for several days. Sulfide concentration in their mixed, undiluted coelomic fluid was 28 μM, and the binding capacity of the fluid was 910 μM. Results of these experiments are shown in Figure 5. The lowest sulfide concentrations tested in these experiments depended on the concentration of blood used in the incubations. At a blood dilution resulting in a sulfide binding capacity of 120 μM, the lowest sulfide concentration tested was 4 μM; at a binding capacity of 360 μM, the lowest sulfide concentration tested was 11.2 μM; and at a binding capacity of 480 μM, the lowest sulfide concentration tested was 14.9 μM. The level of total sulfide (bound and unbound) that inhibited carbon fixation was dependent on the concentration of the blood used in the incubations and was always above the binding capacity of the dilute blood (Fig. 5). Inhibition of carbon fixation in the parallel saline incubations occurred below 350 μM sulfide, and the carbon fixation rates were generally higher when the homogenates were incubated in blood.

Discussion

Potential symbiont substrates

Early work with trophosome preparations, as well as bacterial isolates from high dilutions of trophosome material, suggested that a variety of different types of symbiotic bacteria might be present in the trophosome of *R. pachyptila* (Jannasch, 1983; Fisher and Childress, 1984; Jannasch and Nelson, 1984; *cf* Jannasch, 1989). Similarly, the recent work of Cary *et al.* (1989), Southward (1988), and Jones and Gardiner (1988) all suggest nonvertical transmission of symbionts (both fertilized eggs and early juveniles appear symbiont-free), a situation which, if true, could facilitate the occurrence of a variety of symbionts in *R. pachyptila*. On the other hand, analysis of the 16S rRNA sequences of the symbionts from two specimens of *R. pachyptila* indicate that at least 90% of the symbionts in each individual are of the same species, and that the symbionts in the two individuals are the same (a unique symbiont constituting less than 10% of the bacterial biomass might not be recognized by this technique) (Distel *et al.*, 1988).

The present study confirms the results of Belkin *et al.* (1986); that symbiont carbon fixation is stimulated by sulfide, but not thiosulfate. Similarly, Wilmot and Vetter (1990) demonstrate that sulfide (and not thiosulfate or sulfite) stimulates oxygen consumption by both trophosome preparations and partially purified symbionts of *R. pachyptila*. Furthermore, our results indicate that neither hydrogen nor ammonia stimulate carbon fixation by trophosome preparations. As a test for methanotrophic symbionts, trophosome material from two worms collected at 13°N on the East Pacific Rise was tested for the ability to oxidize ^{14}C-methane, again with negative result. The present study does not directly address the question of symbiont heterogeneity in vestimentiferans, however it does indicate that *R. pachyptila* symbionts from all individuals analyzed are similar in that they use only sulfide as an electron donor to fuel chemoautotrophic carbon fixation.

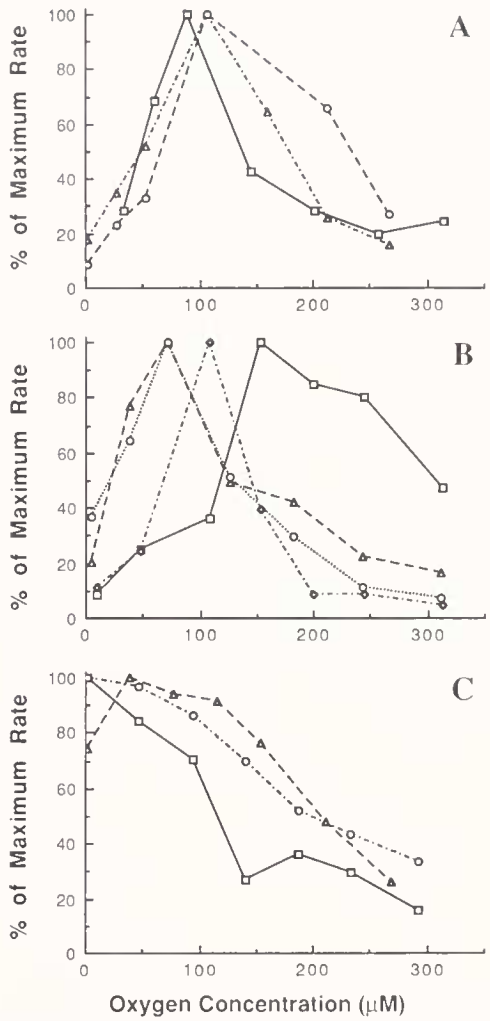

Figure 3. Rate of carbon fixation by *Riftia pachyptila* trophosome preparations as a function of free (unbound) oxygen concentration in three incubation media. Conditions for each incubation are described in Table II. (A) Incubations in saline: triangles, saline #1; circles, saline #2; squares, saline #3. (B) Incubations in 8% *Calyptogena magnifica* serum in saline (sulfide binding capacity, 675 μM): squares, serum #1; triangles, serum #2; circles, serum #3; diamonds, serum #4. (C) Incubations in dilute *Riftia* blood in saline: squares, blood #1; circles, blood #2; triangles, blood #3.

Because of the limited number of experiments conducted previously to test for nitrogen fixation by trophosome preparations (Fisher and Childress, 1984), several additional experiments were conducted with material from the Galapagos Rift. These experiments were conducted under several concentrations of both oxygen and sulfide, and under no condition was there any evidence of nitrogen fixation (acetylene reduction). These results agree with earlier, similar experiments (Fisher and Childress, 1984), enzyme activity measurements (Felbeck, 1981), measurements of $\delta^{15}N$ in *R. pachyptila* tissue (Rau, 1981; Fisher *et al.*, 1988b), and changes in *in situ* nitrate concentration around vent animals (Johnson *et al.*, 1988a). All of these suggest that nitrate is the nitrogen source for the intact symbiosis.

Pressure effects

The results of this study indicate that *Riftia pachyptila* symbionts are barotolerent but not barophilic, although pressure did slightly affect the carbon fixation rate of trophosome preparations (discussed below). These results agree, in general, with ones showing that oxygen consumption by trophosome preparations was not affected by pressure of 100 atm (Fisher and Childress, 1984), and with the fact that all free-living sulfur-oxidizing bacteria isolated from hydrothermal vent waters (and tested) are barotolerant (Jannasch, 1989). Although the actual environmental pressure for these organisms is about 250 atm, 100 atm was considered sufficient for this test because this is enough pressure to keep the tubeworm hosts, as well as other barophilic vent animals, alive (Mickel and Childress, 1982a, b; Arp *et al.*, 1984; Childress *et al.*, 1984).

One effect of increasing pressure is on the dissociation constant for H_2S. Higher pressure favors dissociation of H_2S because of the negative volume change associated with this reaction in water ($\Delta V = -16.3$ cm^3 mol^{-1}) (Isaacs, 1981). Although H_2S is probably the species that crosses cell surfaces (since it is uncharged) and is the species toxic to cytochromes (Smith and Gosselin, 1979), HS^- is the species bound by the hemoglobins of *R. pachyptila* (Childress *et al.*, 1984; Arp *et al.*, 1987). Pressure will also affect the abundance of total sulfide because, by changing relative abundances of charged and uncharged species, it will affect the rate of abiotic oxidation of sulfide. We believe a combination of these variables is responsible for the inconsistent effect of pressure on carbon fixation by the preparations in saline.

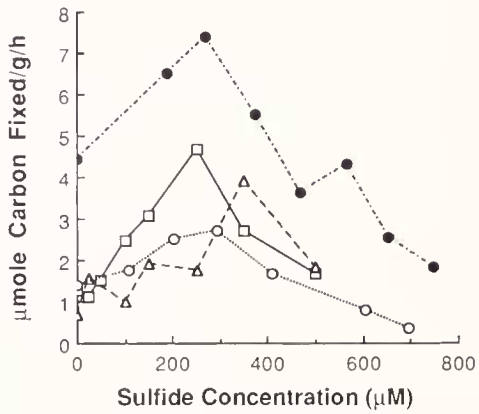

Figure 4. Rate of carbon fixation by *Riftia pachyptila* trophosome preparations in saline as a function of initial sulfide concentration. Each different symbol and line represents a separate experiment.

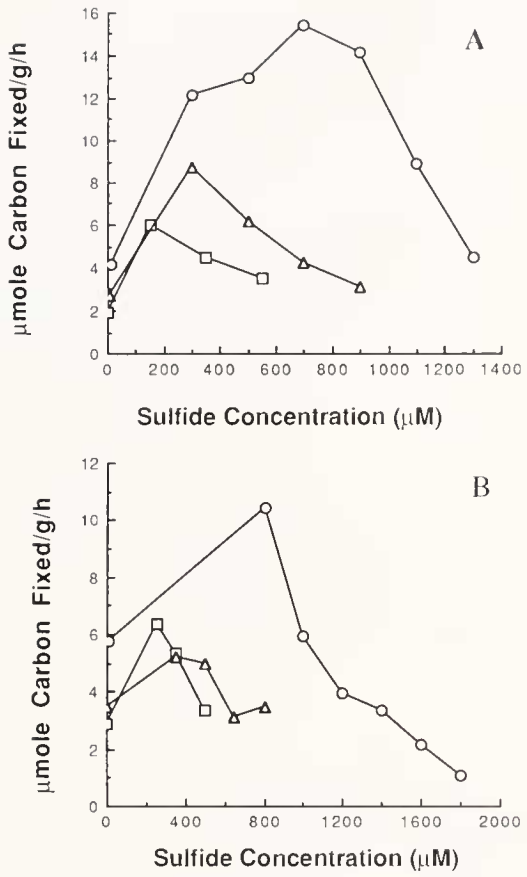

Figure 5. Rate of carbon fixation by *Riftia pachyptila* trophosome preparations as a function of initial total sulfide concentration. A and B represent two separate experiments with trophosome preparations from different worms. Incubations under all three conditions in each experiment were conducted simultaneously. Squares are rates from incubations in saline without added blood; triangles are rates from incubations in dilute (13%) fresh coelomic fluid with a binding capacity of 120 μM sulfide; circles are rates from incubations in more concentrated (53% in A and 40% in B) fresh coelomic fluid with a binding capacity of 360 and 480 μM sulfide, respectively.

The effects of pressure on the blood incubations are also slight. It significantly inhibited carbon fixation at lowest sulfide concentration tested (55 μM), with only slight effects at sulfide concentrations above that, and no effect at concentrations that were clearly inhibitory to carbon fixation. These small effects could be due to either a direct pressure effect on blood sulfide binding or an indirect effect stemming from the altered equilibrium between HS^- and H_2S.

Effects of oxygen concentration on carbon fixation

The possibility that vestimentiferan endosymbionts might be sensitive to free (unbound) oxygen is suggested by the observation that many free-living sulfur bacteria are microaerophilic (Krieg and Hoffman, 1986). Although Wilmot and Vetter (1990) found no oxygen inhibition of oxygen consumption by trophosome preparations, their study does not address the possibility that oxygen may inhibit autotrophic carbon fixation by the symbionts, as oxygen consumption (or sulfide oxidation) and carbon fixation are not tightly coupled in sulfur bacteria (Kelly, 1989). Experiments designed to explore the sensitivity of vestimentiferan symbiont carbon fixation to oxygen were conducted in *Riftia* saline. In these experiments, the maximum rate of carbon fixation was recorded at oxygen concentrations of approximately 100 μM (Table II, Fig. 3A), but the shape of the curves (carbon fixation *vs.* time) suggested increasing substrate limitation over time at the lower oxygen concentrations (Fig. 2A). Due to the reactivity of sulfide and oxygen, either of these substrates could have become limiting during the experiments under those conditions. In a first attempt to stabilize the sulfide without affecting the free oxygen in the experiments, incubations were conducted in dilute *Calyptogena magnifica* serum. This serum was used because it binds sulfide but not oxygen (Arp *et al.*, 1984). The results of the experiments in dilute *C. magnifica* serum were essentially the same as those in saline alone (Table II, Fig. 3B). This suggests that oxygen—not sulfide—is the limiting substrate.

With variable substrate limitation removed from the experimental design by the presence of *Riftia* hemoglobin, the effects of free oxygen on the carbon fixation rate of the trophosome preparations were easily visible (Fig. 3C). At free oxygen levels around air saturation (220 μM), carbon fixation by the trophosome preparations was inhibited by 60 to 70%, while at the maximum ambient oxygen levels the animals are exposed to *in situ* (100 μM), inhibition ranged from about 10 to 40% (Fig. 3C).

Oxygen inhibition of carbon fixation may be caused by the well-documented oxygenase function of the primary carboxylating enzyme in the symbionts, ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBPC/O, EC 4.1.1.39) (Tabita, 1988). However, the experimental conditions of high total inorganic carbon concentration used in these experiments (Table II) would minimize that effect, especially at the lower oxygen concentrations. The predicted inhibition of carbon fixation due to the oxygenase function of RuBPC/O can be calculated using a substrate specificity factor, which compares the relative rates of the carboxylase and oxygenase reactions at any given concentrations of CO_2 and O_2 (Tabita, 1988). The specificity factor has not been experimentally determined for RuBPC/O of *Riftia pachyptila* symbionts, but a factor of 50 is in the range of the lowest values reported for prokaryotic RuBPC/O with both large and small subunits (Tabita, 1988); *R. pachyptila* symbiont RuBPC/O contains both large and small subunits, (Stein *et al.*, 1989). Using this factor to predict the percent of oxygen

inhibition of carbon fixation due to the oxygenase function of the enzyme (RuBPC/O) would, therefore, result in a maximum estimate. Based on the free CO_2 concentrations [calculated from the ΣCO_2 concentrations measured in the experimental syringes (Table II), our experimentally determined relationship between P_{CO_2} and ΣCO_2 in the saline, and the solubility of CO_2 in the saline], we predict no more than 8.4% inhibition of carbon fixation at 220 μM oxygen at the lowest CO_2 concentration employed (in the serum incubations, Table II). A range of 2.5 to 5.7% inhibition by 220 μM O_2 in the other incubations is due to RuBPO. The same calculations predict between 1.2 and 4.2% inhibition of carbon fixation by 110 μM O_2 due to the oxygenase function of RuBPC/O in these experiments.

The most probable explanation for the degree of oxygen inhibition of carbon fixation found in this study is that the symbionts, like many free-living microaerophiles, are sensitive to toxic forms of oxygen, such as H_2O_2, O_2^- or $OH\cdot$ (Krieg and Hoffman, 1986), even though activity of some detoxifying enzymes has been demonstrated in extracts of *R. pachyptila* trophosome (Blum and Fridovich, 1984). Catalase is not present in *R. pachyptila* trophosome, but moderate levels of peroxidase have been demonstrated in trophosome extracts and might defend against H_2O_2 (Blum and Fridovich, 1984). Activity of superoxide dismutase against superoxide ions has also been demonstrated in *R. pachyptila* trophosome, but the activity was substantially lower than that in muscle tissue. Furthermore, the activity was nonlinear after less than 1 min in cell-free preparations (Blum and Fridovich, 1984), and, therefore, may be inactive in our longer term trophosome experiments. Several microaerophiles contain activities of these protective enzymes and yet are sensitive to toxic forms of oxygen (reviewed by Kreig and Hoffman, 1986). One possible explanation for this is that intracellular protective enzymes of the microaerophile *Campylobacter fetus* are apparently ineffective against exogenous O_2^- and H_2O_2, which could be adversely affecting the microbial cell surface (Hoffman *et al.*, 1979). The subcellular location of the protective enzymes in *Riftia* symbionts is unknown. The possibility that symbionts are sensitive to toxic forms of oxygen is further substantiated by three other observations. (1) Exposure to moderate levels of oxygen during preparation of a trophosome homogenate substantially lowers the activity of the preparation. (2) Highest rates of carbon fixation in saline incubations occurs when homogenates were prepared under virtually anaerobic conditions (Table II). (3) When the same preparation is incubated in dilute *Riftia* blood and in saline, the incubations in blood fix carbon at consistently higher rates. Unequivocal demonstration of symbiont sensitivity to toxic forms of oxygen awaits studies that demonstrate relief of oxygen inhibition by substances that quench toxic forms of oxygen (see review by Kreig and Hoffman, 1986).

Whatever the cause of the observed reduction in carbon fixation rate as a function of free oxygen concentration, the role of blood in mitigating this effect, while providing an abundant pool of oxygen, is evident (Table II, Figs. 2, 3). That the highest carbon fixation rates were observed when free oxygen in solution approached zero (the P50 of *Riftia* hemoglobin is around 2 μM at 7.5°C; Arp and Childress, 1981) indicates that the symbionts have an even higher affinity for oxygen and can remove it from the blood. The maximum levels of free oxygen possible in the blood of living worms *in situ* would be equal to the maximum oxygen levels in the surrounding water—110 μM in the ambient (non-vent) bottom water (Johnson *et al.*, 1988a). According to detailed surveys of oxygen and sulfide conditions at the central clump of *R. pachyptila* at the Rose Garden vent on the Galapagos Rift, the worms are exposed to levels of oxygen that vary from 110 μM to undetectable over very short or moderate time scales (Johnson *et al.*, 1988b). Thus, levels of oxygen in blood fresh from the plume would be expected to vary considerably. The anatomy of *Riftia* and the properties of its blood buffer its symbionts from the extremes in ambient oxygen concentration (which range from limiting to inhibitory), and allow maximal rates of carbon fixation by providing the symbionts with an abundant pool of oxygen, while maintaining low levels of free (unbound) oxygen in the trophosome.

Effects of sulfide concentration on carbon fixation

The experiments designed to address this question can be divided into three groups: experiments in saline; experiments in mixed blood with reduced sulfide binding capacity; and comparative experiments in saline and fresh coelomic fluid.

The maximum rates of carbon fixation in experiments in saline occurred at initial sulfide concentrations between 250 and 350 μM (Fig. 4). The differences in the sulfide concentrations yielding maximum carbon fixation rates could be due to real differences in sulfide sensitivity of the symbionts from different individuals. However, the inherent instability (and unpredictability) of unbound sulfide and oxygen in solution cast some doubt as to the actual levels of sulfide present during these incubations; the apparent differences could be due to differing rates of auto-oxidation of sulfide in the different incubations and resultant variations in actual sulfide concentrations during the incubations.

Two general conclusions stand out from the data collected in the incubations with blood of reduced binding capacity. First, even this blood protects the symbionts

from the inhibitory effects of sulfide demonstrated in the saline incubations (Fig. 4). Second, carbon fixation by *R. pachyptila* symbionts is not inhibited by levels of sulfide below the binding capacity of the blood.

In the experiments with fresh coelomic fluid, as found in the other experiments reported here and in previous work (Fisher *et al.*, 1988a), the blood protected the symbionts from the inhibitory effects of sulfide at sulfide concentrations below the binding capacity of the blood. The maximal rates of carbon fixation in the blood incubations were also higher than the maximum rates in the corresponding saline incubations. However, unlike the symbionts of a hydrocarbon seep escarpid vestimentiferan which were inhibited by total sulfide concentrations greater than 70% of capacity (Fisher *et al.*, 1988a), *Riftia pachyptila* symbionts were not inhibited by sulfide until the concentration surpassed the binding capacity of the dilute blood (Fig. 5). The exact level of free sulfide that was inhibitory to *R. pachyptila* symbionts cannot be determined due to the paucity of data points around the carbon fixation maxima (Fig. 5). However, it is clearly above the 100 μM that is inhibitory to the seep escarpid (Fisher *et al.*, 1988a), and more likely closer to 350 μM as suggested by the saline incubations (Fig. 4). Another recent study (conducted during the same cruise as these experiments) demonstrated that oxygen consumption by trophosome preparations was not inhibited by sulfide concentrations up to 2 mM (Wilmot and Vetter, 1990). The results of that study and this one are not contradictory; most likely they reflect the relatively "loose" coupling between sulfide oxidation and carbon fixation, and indicate that trophosome preparations can oxidize sulfide without concomitant carbon fixation. An earlier study of sulfide stimulation of carbon fixation by *Riftia* symbionts showed an optimum of 600 μM, however, these workers' trophosome preparations had very low levels of activity and apparently no effort was made to exclude or quantify blood contamination in the preparations (Belkin *et al.*, 1986). Thus, while their results are in general agreement with ours, a precise comparison cannot be made.

The lesser sensitivity to free sulfide of autotrophic carbon fixation by the symbionts of *R. pachyptila*, as compared to the seep escarpid, is probably a reflection of the ambient sulfide levels the animals are exposed to in their respective environments. The highest sulfide levels measured around the plumes of the seep escarpid are below 3 μM, and even if these vestimentiferans take up sulfide across their body wall, as has been suggested (MacDonald *et al.*, 1989), the symbionts are likely never exposed to sulfide levels in the blood approaching saturation. In fact, the highest level reported in the blood of freshly collected seep vestimentiferans is 114 μM (Childress *et al.*, 1986) which, assuming the blood has similar capacities to *R. pachyptila* blood, corresponds to a free sulfide concentration below 1 μM (Fisher *et al.*, 1988a). This assumption is validated by preliminary experiments with blood from the seep escarpid, which indicate that the blood binds sulfide and is of similar concentration to that of *R. pachyptila* (A. J. Arp, pers. comm.). *R. pachyptila* is exposed to sulfide levels in the 300 μM range *in situ* (Johnson *et al.*, 1988a), and total sulfide levels in the blood of freshly collected *R. pachyptila* as high as 9 mM have been reported (Childress *et al.*, 1984). Therefore, free sulfide levels in the blood of *R. pachyptila* are apt to be much higher than those found in the seep escarpid, and *R. pachyptila* symbionts are apparently adapted to these higher concentrations.

The symbionts of both species are located in vacuoles within host cells (bacteriocytes) in the trophosome, and not directly exposed to the blood *in situ*. The conditions inside the vacuole and host bacteriocyte will certainly affect both the total amount of sulfide and the ionic species to which the symbionts are exposed. However, the high degree of vascularization of the trophosome (Jones, 1988) implies that the blood exerts considerable influence on the concentrations of sulfide and oxygen in the bacteriocytes, and that the maximum concentrations to which the symbionts are exposed are almost certainly a reflection of the highest free concentrations of these substances in the blood.

Despite the differences between the species, the role of the blood with respect to sulfide in the intact *R. pachyptila* symbiosis is basically the same as for the seep escarpid (Fisher *et al.*, 1988a). That role is to provide the symbionts with an abundant supply of sulfide, while maintaining free sulfide at levels that allow maximal rates of carbon fixation.

Conclusion

R. pachyptila individuals appear to grow rapidly (Fustec *et al.*, 1987; Hessler *et al.*, 1988; Roux *et al.*, 1989). Because these animals are apparently dependent upon their symbionts for at least their bulk nutritional carbon requirements (see review by Fisher, 1989), the trophosome must be a very productive chemoautotrophic organ. Shipboard studies with live animals under pressure suggest that the oxygen consumption rate of *R. pachyptila* is in the range of 0.44 to 1.52 μmole oxygen g^{-1} h^{-1} in the absence of sulfide (Childress *et al.*, 1984). Assuming a RQ of 1, these data suggest that the intact symbiosis requires an input of organic carbon at about the same rate. Since the trophosome accounts for 15.3 ± 4.9% of the worms wet weight (Childress *et al.*, 1984), the trophosome must incorporate inorganic carbon into organic compounds at at least 10 μmole carbon g trophosome^{-1} h^{-1} to meet the metabolic needs of the intact symbiosis.

The higher rates reported in this study (20 to 28 μmole C g^{-1} h^{-1}) suggest that the symbionts can meet the bulk nutritional carbon requirements of the intact symbiosis through chemoautotrophy, even assuming an efficiency of 50% or less in the transfer of nutritional carbon from symbiont to host. This calculation also supports the contention that the activities of the preparations used in this study are reasonable, and that preparations with significantly lower activity are suboptimal.

The stable carbon isotope composition of these animals has been interpreted as reflecting carbon limited symbionts (Rau, 1981, 1985; Fisher *et al.*, 1988c). This interpretation implies a high rate of consumption of inorganic carbon by the symbionts and also suggests a trophosome with high metabolic activity (both of which are supported by this study). The high sulfide and oxygen binding capacities of the hemoglobins of *R. pachyptila* are therefore necessitated by both the relatively large quantity of trophosome and its high autotrophic capacity. Additionally, the affinity of the blood for sulfide and oxygen allows the symbionts access to these large pools of bound substrates without exposing the symbionts to high free concentrations of either substance, thereby supporting maximal rates of carbon fixation by the symbionts.

Vestimentiferans rely on a finely tuned symbiosis for their survival. Both their anatomy and the properties of vestimentiferan hemoglobins are adapted for symbiosis with a specific type of sulfide-oxidizing symbiont. The host tube-worms reap the benefits of an autotrophic life style, while providing their symbionts with an environment which free-living sulfide-oxidizing bacteria can only regard with envy.

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